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**Weyens et al.**

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(54) **T227-1 FLANKING SEQUENCE**

(75) Inventors: **Guy Weyens**, Beersel (BE); **Steve Barnes**, Petit-Hallet (BE); **Inge Rosquin**, Lier (BE)

(73) Assignee: **SES Europe N.V./S.A.**, Tiémen (BE)

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(51) **Int. Cl.**  
**C12Q 1/68** (2006.01)

(52) **U.S. Cl.** ..... **435/6; 435/91.1; 435/91.2**

(58) **Field of Classification Search** ..... None  
See application file for complete search history.

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*Primary Examiner*—Jeffrey Fredman

*Assistant Examiner*—Heather G. Calamita

(74) *Attorney, Agent, or Firm*—Christensen O'Connor Johnson Kindness PLLC

(57) **ABSTRACT**

A herbicide resistant transformed sugar beet that is detectable by the specific primers developed to match the DNA sequences that flank the left and/or right border region of the inserted transgenic DNA and the method of identifying primer pairs containing plant genomic DNA/plasmid DNA. More specifically the present invention covers a specific glyphosate resistant sugar beet plant having an insertion of the transgenic material identified as the T227-1 event. The present invention additionally covers primer pairs: plant genomic DNA/Plasmid DNA that are herein identified. Additionally, these primer pairs for either the left or the right flanking regions make an event specific test for the T227-1 insert of transgenic material.

**6 Claims, 12 Drawing Sheets**

FIGURE 1: Transformation event T227-1 with positions of the specific primers

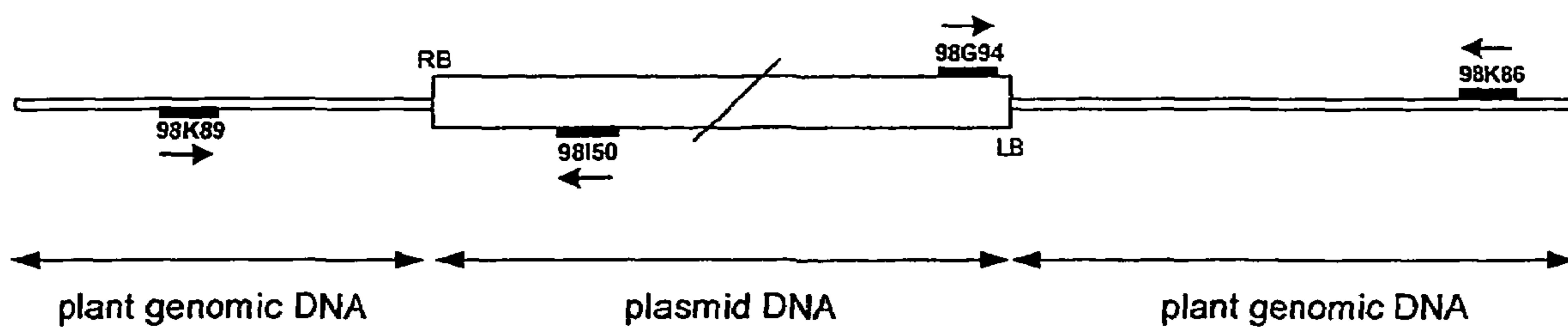


FIGURE 2  
Diagrammatic interpretation of the Southern blot results.

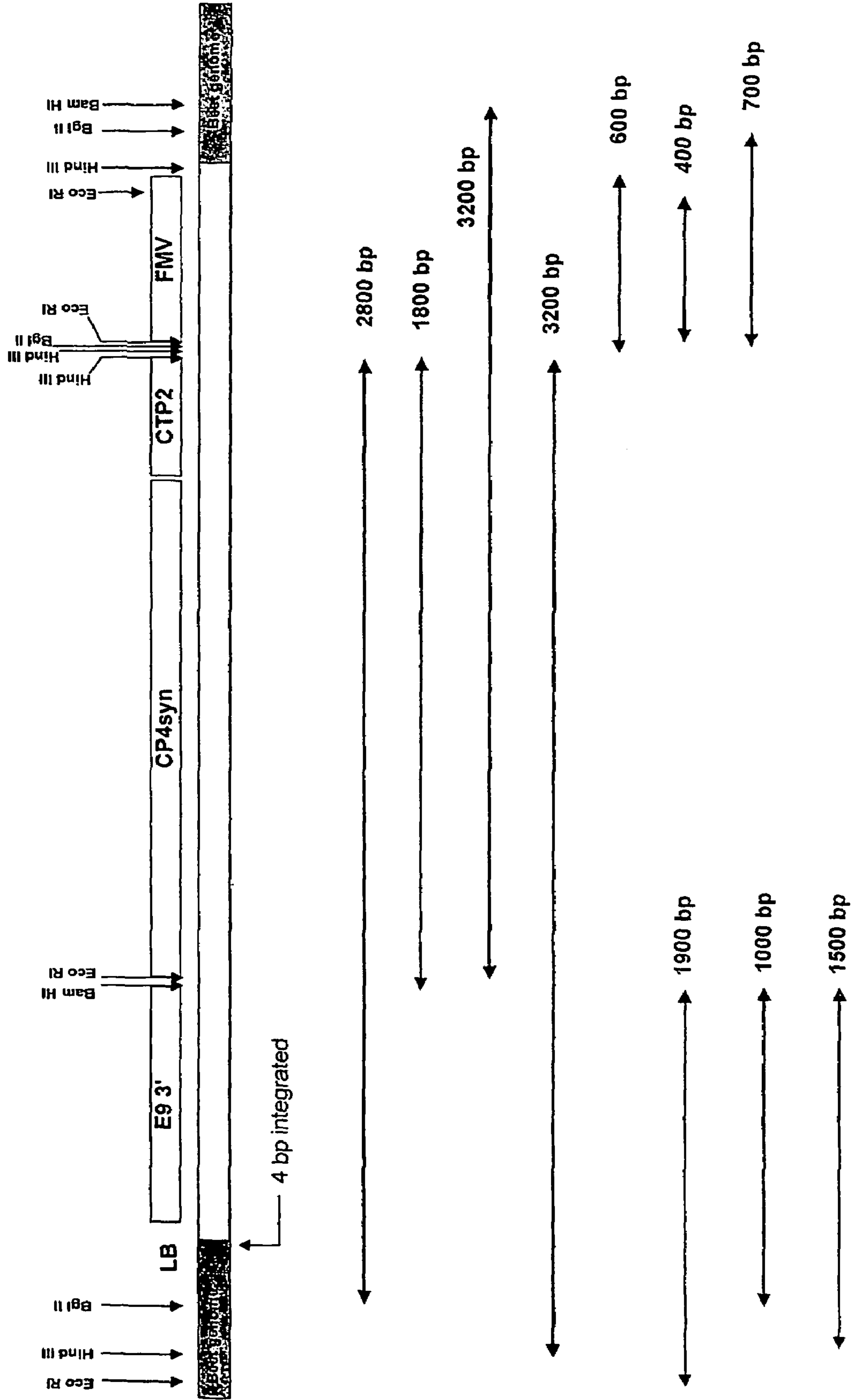
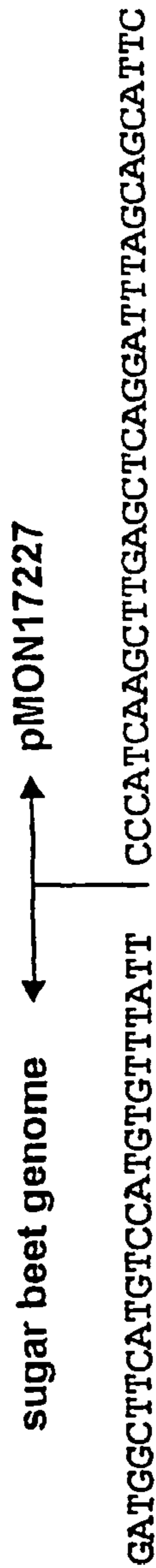


FIGURE 3  
Plasmid sequence at the right border.

RIGHT BORDER



(SEQ ID NO: 1)

Plasmid  
sequence  
(bp)

SUB106

7800 CCTG**TGGTTG GCATGCACAT ACA**AATGGAC GAACGGATAA ACCTTTTCAC

(SEQ ID NO: 2)

7850 GCCCTTTTAA ATATCCGTTA TTCTAATAAA CGCTCTTTTC TCTTA GGTTT

RB7

7900 **ACCGCCAAT ATATCCTGTC** AAACACTGAT AGTTTAAACT GAAGGCGGGA

Break at 7985 bp

RB11

RB12

7950 AACGACAATC TGATC**CCCAT CAAGCTTGAG** **CTCAGGATTT AGCAGCATTC**

SUB106

8000 ...219bp... **GCATCATGGT CAGTAAGTTT CAGAAAAA**

(SEQ ID NO: 3)

FIGURE 4  
 Sequence of the right border breakpoint. (SEQ ID NO: 4)

Sugarbeet	GGACAGGACA	CGACAGCTCC	TCACGAGGTA	ATGGATATCA	TTAGAGAAAG	50
genomic	AGCGGAACAA	ATATTACTCA	AATGGAGGAT	TTATGAAAGT	AATAGATATA	100
sequence	CTTTACTAGA	AAAGGAAGAT	TGTCATGATT	CAACACCAA	TGACACTTAA	150
98K89 primer	<b>ATTA<sup>8</sup>AGAACC</b>	<b>CACCTCATCT</b>	<b>TAAACCAAA<sup>11</sup>C</b>	TAAAATATCA	TTTAATACAT	200
	ATCCAAGTCA	TAATCTACTA	GTAGTTTTC	TTGGTGAGAT	TACATAATAT	250
<b>BglII</b> restriction site	ATCACTAATA	TATAAGAAAT	TTATTTTTC	ATCA <b>AGATCT</b>	ATACAACATA	300
	TAACTGAAGT	AGGAGAAGAT	ATGGGATTGG	TGTGGGAGAT	GGCTTCATGT	350
Integrated plasmid	CCATGTGTTT	ATCCCATCA	<b>AGCTTGAGCT</b>	<b>CAGGATTAG</b>	<b>CAGCATTCCA</b>	400
sequence			<i>HindIII</i> restriction site		<b>RB12 primer</b>	

FIGURE 5

Alignment of the right breakpoint region and the untransformed plant allelic sequence

```

UNTRANSF      ATTTAAATRTC
                * * * * *
T227-1 right  AATTAAGAACCCACCTCATCTTAAACCAAACTAAATATC

UNTRANSF      WKTTGMCACATATCCAAGTCATAATCTACKASYAGTTTKG
                * * * * *
T227-1 right  ATTTAATACATATCCAAGTCATAATCTACTAGTAGTTTGG

UNTRANSF      CTTGGTGAGATTACATAATAKATCACTAATATAASAAA
                * * * * *
T227-1 right  CTTGGTGAGATTACATAATATACACTAATATAAAGAAA

UNTRANSF      TTTATTTWTCAATCAAGATCTATACAACATAATWMCTGAAG
                * * * * *
T227-1 right  TTTATTTTTC AATCAAGATCTATACAACATAAATAACTGAAG

UNTRANSF      TAGGAGAAGATATGGGATTGGTGGGASATGGATTCCCC
                * * * * *
T227-1 right  TAGGAGAAGATATGGGATTGGTGGGAGATGGCTTCATG

UNTRANSF      ATATAAGTAAAGAGAGTCAA

T227-1 right  TCCATGTGTTTATTCCCATCAAGCTTGAGCTCAGGATTTA
                17bp fragment      plasmid sequence

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FIGURE 6  
Plasmid sequence at the left border.

LEFT BORDER

pMON17227 ←      → sugar beet genome

CTGATCCATGTAGATTTCCCGGACATGAAGCCATT | CCATATCTTCCTACTTTC

(SEQ ID NO: 7)

Plasmid  
sequence  
(bp)

SUB103

AATTGATTGAC AACATGCATC AATCGACCTG ...284bp...

(SEQ ID NO: 8)

LB8

Break at 2686 bp

CTGATCCATG TAGATTTCCC GGACATGAAG CCATTTACA TTGAATATAT

LB9

CCTGCCGCCG CTGCCGCTTT GCACCCGGTG GAGCTTGCAT GTGGTTTCT

SUB104

ACGCAGAACT GAGCCGGTTA GGCAGATAAT TTCATGAG AACTGAGCCA

TGTGCACCTT (SEQ ID NO: 9)

FIGURE 7

Sequence of the left border breakpoint. (SEQ ID NO: 10)

Integrated plasmid sequence LB8 primer	AATGfNCTfF GANAAAAAAT CTCATTGNTG CTTCCTCCTAC ATATTTGTCC TCGAATTTTC GCAATGAGCG GAATGCATCG AATCAAGCTA TGTCGATGCC AATTGGTTAC CATGGAATA ATGCGAGTCC AATTTCGCTC TGATACCAGA GTTGTCTCTT	CATTTTATAA TGGTAAFTAC ATCCATGTAAG TTCNAGTCNA CAACTCTCTT TTCCTAATAA AGAAATANAA TCCTACTGTG TGCTPATTC ATGGGTTTTT CATAGAACCCT AAAAGGGTAG CGACCCTACA GAACTCATAA TATTCCTATG AGTTTCAATG	NAAGGCTGCG TCTTTCTTTT ATTCCCGGA TTAGTTGTAT TTATTTCCCTG TCTCGCGATA ATTTTATTTC ATTGCATGAG ATCTGCTCTG TAGGGGGAAT ATCATTAGAC GGCTATTCT AAACGCCAAG TCAGCTTGG <sup>3'</sup> AAAGGAATA <sup>3'</sup> ATTGGGTNNN	GACATCTACA TCGCCATATN CATGANGCCA AGATCTTGAT TGTCCATGTC ACTTGCATGG TGCTTTGAAA TGGAAACACA GGGTACTCTG TTTGATTTAT ATGCATGATC TTGGTATTTC TTCCTTGGCA CTCATAGCAG TGCCTTGTTCG TGACATGGAT	TTTTTGAATT GACCATCATA TTTCCCATAT TGAAAAATAA TGAACAACCTT TTTGGAACAT GCAATTGTTA TATGGGAGGA GTCATACTCG TGGAAACCAG AACATATAAC TCAGCGGTTT AGAATTAAAG GAGATTTTAA GAAACTCAAC TAATGAA	50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 797
<b>BglII restriction site</b>						
Sugarbeet genomic sequence						
<b>HindIII restriction site</b>						
<b>98K86 primer</b>						



FIGURE 8

Alignment of the left breakpoint region and the untransformed plant allelic sequence.

```

UNTRANSF          ATATGGGAT-TGGTGTGGGASATGGATTCCCCATAT
                   *** ** * * * * * * * * * *
T227-1 left  AAGCCATTTCCCATATCTTCTCCTACTTCNAGTCN—AT
                plasmid sequence                49bp fragment

UNTRANSF          AAAGTAAAGAGAGTCAACAAGAAGAWATAAAATATTTGTCC
                   * * * * * * * * * * * * * * * * * * * * * *
T227-1 left  TAGTTGTATAGA-TCTTGATTGAAAAATAAA-TATTTGTCC

UNTRANSF          CAACTCTCTTTTATYCC-TGTGTCCATGTCTGAACAACCTYT
                   *****
T227-1 left  CAACTCTCTTTTATTCCCTGTGTCCATGTCTGAACAACCTT

UNTRANSF          CGAATTTTCTTCCTAATAATCTCGCGATAACTTGCATGGTT
                   *****
T227-1 left  CGAATTTTCTTCCTAATAATCTCGCGATAACTTGCATGGTT

UNTRANSF          TGGAACATGCAATGAGCGAGAAATAGAAATTTTATTTCTGC
                   *****
T227-1 left  TGGAACATGCAATGAGCGAGAAATANAAATTTTATTTCTGC

UNTRANSF          TTTGAAAGCAATTGTTAGAATGCATCGTCSTACWGTGATTG
                   *****
T227-1 left  TTTGAAAGCAATTGTTAGAATGCATCGTCCTACTGTGATTG

UNTRANSF          CATGAGTGGAACACATATGGGAGGAAATCAASCTATGTCT
                   *****
T227-1 left  CATGAGTGGAACACATATGGGAGGAAATCAAGCTATGTCT

UNTRANSF          ATTGCATCTGCTCTGGGGTACTCTGGTCATACTCGTGTCTGA
                   *****
T227-1 left  ATTGCATCTGCTCTGGGGTACTCTGGTCATACTCGTGTCTGA

UNTRANSF          TGCCATGGGTTTTTTAGGGGGAATTTTGATWTATTGGAAAC
                   *****
T227-1 left  TGCCATGGGTTTTTTAGGGGGAATTTTGATTATTGGAAAC
    
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UNTRANSF      CAGAATTGGKWRCATAGAACCTATCATTAGACATGCATGA
                *****
T227-1 left   CAGAATTGGTTACCATAGAACCTATCATTAGACATGCATGA

UNTRANSF      TCAACATATAACCATGGAAATAAAAAGGGTWGGGGCTATTC
                *****
T227-1 left   TCAACATATAACCATGGAAATAAAAAGGGTAGGGGCTATTC

UNTRANSF      CTTTGGTATTTCTCAGCGGTTTATGCGAGTCCSGACCCTAC
                *****
T227-1 left   -TTTGGTATTTCTCAGCGGTTTATGCGAGTCCCGACCCTAC

UNTRANSF      AWAACGCCAAGTTACTTTGGCAAGAATTAAGAAATTTGCT
                *****
T227-1 left   AAAACGCCAAGTT-CTTTGGCAAGAATTAAGAAATTTGCT

UNTRANSF      CGAACTCATWMTCAMGCTKSGCTCATRGCMGAGAWTTTAA
                *****
T227-1 left   CGAACTCATAATCAAGCTTGGCTCATAGCAGGAGATTTTAA

UNTRANSF      TGWK-CCARATKBCCTATGAAAGGAAA
                **** *
T227-1 left   TGATACCAGATATTCCTATGAAAGGAATACTGCTTGTTTCGG
    
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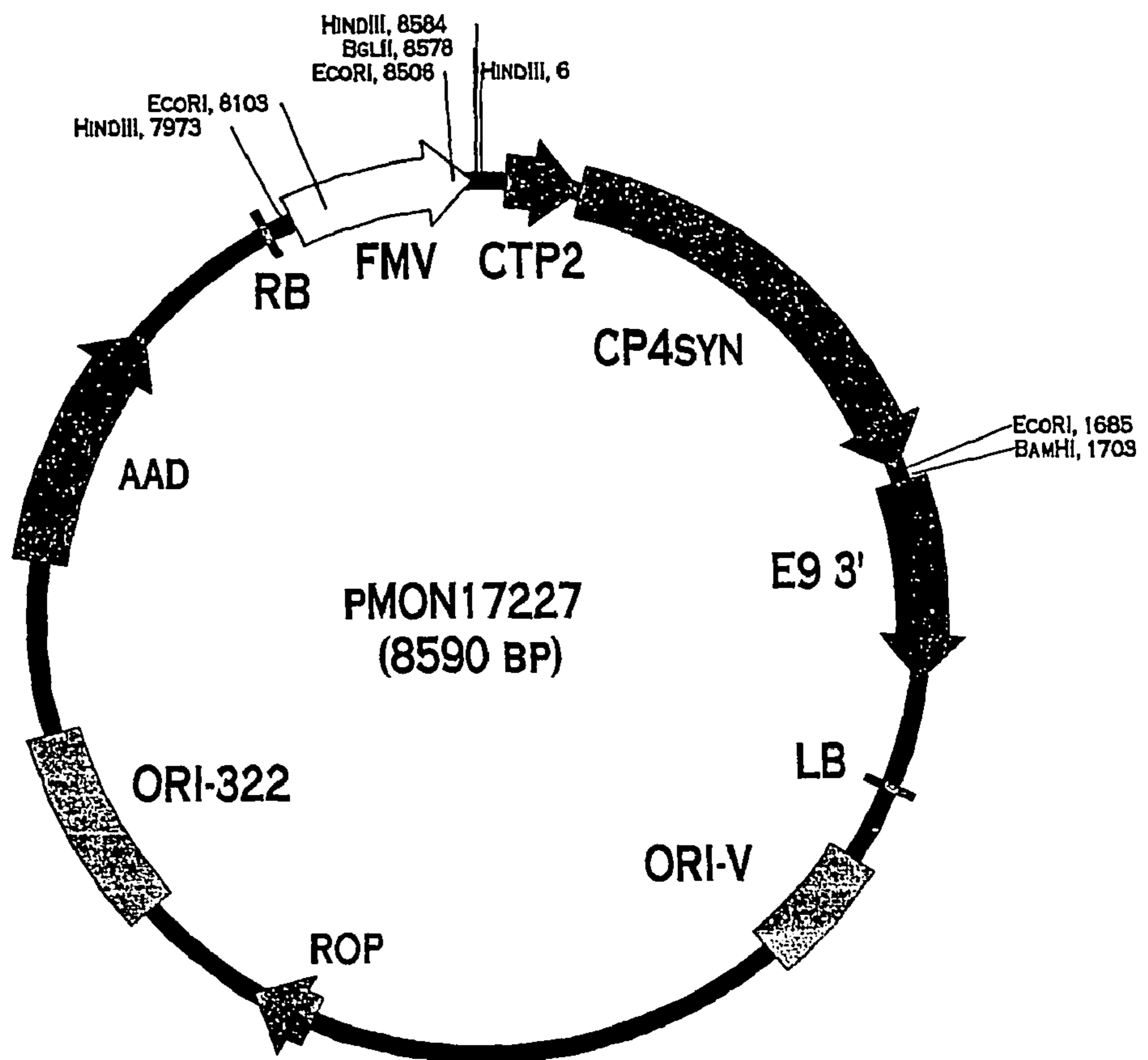
Legend:

UNTRANSF - SEQ ID NO:11

T277-1 right - SEQ ID NO:12

FIGURE 8 (Continued)

FIGURE 9 Restriction sites of the enzymes on pMON17227.



**Figure 10a****Sequence of the right border fragment obtained with the 98K89 – RB12 primer set.****(SEQ ID NO: 13)****98K89 primer**

**AGAACCCACC TCATCTTAAA CCAAAC TAAA ATATCATT TA ATACATATCC 50**  
**AAGTCATAAT CTACTAGTAG TTTTGCTTGG TGAGATTACA TAATATATCA 100**  
**CTAATATATA AGAAATTTAT TTTTCAATCA AGATCTATAC AACTAATAAC 150**  
**TGAAGTAGGA GAAGATATGG GATTGGTGTG GGAGATGGCT TCATGTCCAT 200**  
**GTGTTTATTC CCATCAAGCT TGAGCTCAGG ATTTAGCAGC ATTCCA 246**

**RB12 primer****Figure 10b****Sequence of the right border fragment obtained with the 98K89 – 98I50 primer set.****(SEQ ID NO: 14)****98K89 primer**

**AGAACCCACC TCATCTTAAA CCAAAC TAAA ATATCATT TA ATACATATCC 50**  
**AAGTCATAAT CTACTAGTAG TTTTGCTTGG TGAGATTACA TAATATATCA 100**  
**CTAATATATA AGAAATTTAT TTTTCAATCA AGATCTATAC AACTAATAAC 150**  
**TGAAGTAGGA GAAGATATGG GATTGGTGTG GGAGATGGCT TCATGTCCAT 200**  
**GTGTTTATTC CCATCAAGCT TGAGCTCAGG ATTTAGCAGC ATTCCAGATT 250**  
**GGGTTCAATC AACAAGGTAC GAGCCATATC ACTTTATTCA AATTGGTATC 300**  
**GCCAAAACCA AGAAGGAACT CCCATCCTCA AAGGTTTGTA AGGAAGAATT 350**  
**CTCAGTCCAA AGCCTCAACA AGGTCAGGGT ACAGAGTCTC CAAACCATTA 400**  
**GCCAAAAGCT ACAGGAGATC AATGAAGAAT CTTCAATCAA AGTAAACTAC 450**  
**TGTTCCAGCA CATGCATCAT GGTCAG 476**

**Figure 10c**

**Sequence of the left border fragment obtained with the 98G94 – 98K86 primer set.  
(SEQ ID NO: 15)**

**98G94 primer**

**CGCCTATAAA TACGACGGAT CGTAATTTGT CGTTTTATCA AAATGTA CTT 50  
TCATTTTATA ATAACGCTGC GGACATCTAC ATTTTGAAT TGAAAAAAAAA 100  
TTGGTAATTA CTCTTTCTTT TTCTCCATAT TGACCATCAT ACTCATTGCT 150  
GATCCATGTA GATTCCCGG ACATGAAGCC ATTTCCATA TCTTCTCCTA 200  
CTTCNAGTCN ATTAGTTGTA TAGATCTTGA TTGAAAATA AATATTTGTC 250  
CCAACTCTCT TTTATTCCCT GTGTCCATGT CTGAACAACT TTCGAATTTT 300  
CTTCCTAATA ATCTCGCGAT AACTTGCATG GTTTGGAACA TGCAATGAGC 350  
GAGAAATANA AATTTTATTT CTGCTTTGAA AGCAATTGTT AGAATGCATC 400  
GTCCTACGTG ATTGCATGAG TGGAAACACA TATGGGAGGA AATCAAGCTA 450  
TGTCTATTGC ATCTGCTCTG GGGTACTCTG GTCATACTCG TGTCGATGCC 500  
ATGGGTTTTT TAGGGGGAAT TTTGATTTAT TGGAAACAGA ATTGGTTACC 550  
ATAGAACCCTA TCATTAGACA TGCATGATCA ACATATAACC ATGGAAATAA 600  
AAAGGGTAGG GGCTATTCTT TGGTATTTCT CAGCGGTTTA TGCGAGTCCC 650  
GACCCTAGAA AACGCCAAGT TCTTTGGCAA GAATTAAGAA ATTTGCTCG 700  
AACTCATAAT CAAGCTTGGC TCATAGCAGG AGATTTTAAT GATACCAGAT 750  
ATTCCTATGA AAGGAATACT GCTTGTTCCG AACTCAACG TT 792**

**98K86 primer**

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**T227-1 FLANKING SEQUENCE****CROSS REFERENCES TO RELATED APPLICATIONS**

This application claims the benefit under 35 U.S. C. §371 as a national filing of the international application Number PCT/GB01/05321 filed 30 Nov. 2001 and claims the benefit of the U.S. provisional application No. 60/250,110 filed 30 Nov. 2000.

**FIELD OF THE INVENTION**

The field of the invention broadly covers a herbicide resistant transformed sugar beet that is detectable by the specific primers developed to match the DNA sequences that flank the left and/or right border region of the inserted transgenic DNA and the method of identifying primer pairs containing plant genomic DNA/plasmid DNA. More specifically the present invention covers a specific glyphosate resistant sugar beet plant having an insertion of the transgenic material identified as the T227-1 event. The present invention additionally covers primer pairs: plant genomic DNA/Plasmid DNA that are herein identified. Additionally, these primer pairs for either the left or the right flanking regions make an event specific test for the T227-1 insert of transgenic material. These event specific tests using the primers include a method that employs electrophoresis through gels as the standard method used to separate, identify and purify DNA fragments. The location of the DNA that are separated by size within the gels can be determined by using a dye. This dye permits DNA bands with 1–10 ng of DNA to be detected under ultraviolet light.

**BACKGROUND**

In the last few years there has been numerous commercial products having glyphosate resistance plants such as soybeans, maize, and rapeseed. At least two different genes have been used to make these commercial products. These products have had a specific event which has been developed through extensive research and testing and then registered with the regulatory authorities in the various countries as genetically modified organisms.

The regulatory requirements in Europe for introduction of plants that have had foreign DNA introduced are most stringent. Although not a specific requirement regulatory offices favor inserted events containing DNA which is necessary for the expression of the introduced trait and very little extra DNA in the insert. On the practical side, the event should have low numbers of copies to avoid problems with gene silencing. Development of these types of insertion events in certain crops such as sugar beets can be difficult. The inserted event should provide the plant with the desired levels of gene expression such as resistance to the application of a herbicide, resistance to insect attack, or production of an oil or sugar, and the like.

Additionally, there is the need to have an insert in sugar beet that is identifiable through tests developed for the inserted event. These test results can be employed to track the GMO in production plants and fields.

There is a need to be able to clearly identify a transgenic plant through its inserted DNA. The need for identifiable transgenic events and the primers and the event specific tests for these primers are increasingly evident.

**SUMMARY OF THE INVENTION**

Broadly the present invention is to a method of detecting a glyphosate resistant sugar beet comprising the steps of

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forming primers that flank the genomic and plasmid sequence border in the sugar beet which can uniquely identify the sugar beet, by using a PCR to sequence the unique fragment produced by the primers and detecting the absence or presence of the fragment. The present invention covers a glyphosate resistant sugar beet detectable by this method. Additionally this invention covers a transgenic glyphosate resistant sugar beet comprising an insertion of DNA unique to T227-1 and its progeny. This invention is detectable by either of the pair of primers for the left or right border. The primer set capable of generating a DNA fragment unique to identify T227-1 of 50 base pairs and more preferably approximately 100 base pairs. Wherein the DNA fragment unique to identify T227-1 includes some base pairs related to the sugarbeet genomic DNA and some base pairs related to the inserted plasmid DNA (from pMon17227 in T227-1. A sugar beet that is detectable with a pair of primers in accordance with the present invention that border the left flanking sequence identified as 98G94 (nucleotides 1–20 of SEQ ID NO: 15) and 98K86 (nucleotides 730–753 of SEQ ID NO: 10) generating a 792 bp fragment (SEQ ID NO: 15).

A sugar beet is also detectable with the pair of primers that border the right flanking sequence identified as 98I50 (nucleotides 455–476 of SEQ ID NO: 14) and 98K89 (nucleotides 155–180 of SEQ ID NO: 4) generating a 476 bp fragment (SEQ ID NO: 14). Or the primer set 98K89 (nucleotides 155–179 of SEQ ID NO: 4)–RB12 (nucleotides 185–200 of SEQ ID NO: 2) can also be employed to produce a unique fragment of 246 base pairs (SEQ ID NO: 13).

The invention also includes the pairs of primers comprising DNA which flank at least one of the border regions of the insertion of DNA into the T227-1 Event.

The T227-1 event is sugarbeet material that is capable of glyphosate resistance when in the plant form and is capable of being detected by at least one of the pair of primers 98G94 (nucleotides 1–20 of SEQ ID NO: 15) and 98K86 (nucleotides 730–753 of SEQ ID NO: 10), 98I50 (nucleotides 455–476 of SEQ ID NO: 14) and 98K89 (nucleotides 155–180 of SEQ ID NO: 4), and/or 98K89 (nucleotides 155–180 of SEQ ID NO: 4)–RB12 (nucleotides 185–200 of SEQ ID NO: 2). The primers should when a positive result is produced identify a DNA fragment or a fragment of a length not associated with the primers. The invention has identified a pair of primers comprising DNA which lies in right border region of insertion of DNA into the T227-1 Event wherein one primer lies in the plant genomic material and the other primer is in the inserted plasmid material.

The invention has also identified a pair of primers comprising DNA which lies in the left border region (FIG. 6) of insertion of DNA into the T227-1 Event wherein one primer lies in the plant genomic material and the other primer is in the inserted plasmid material.

The primers themselves are generated to identify the T227-1 event by employing a PCR test using a pair of primers. The primers are used in a PCR method to detect the presence or absence of the T227-1 event. A test kit for the T227-1 event can be formed with the primers as components thereof.

The method of detecting the T227-1 event including the steps of selecting sugar beet genomic material for testing; employing at least one of the pair of primers capable of detecting the T227-1 event in association with such selected material; and using a PCR machine to amplify the DNA fragment if it exists; detecting the presence or the absence of the DNA fragment.

The transgenic glyphosate resistant sugar beet of the present invention is characterized by a unique sequence of DNA having at least 80% homology to the right border

sequence in FIG. 3 (SEQ ID NO: 2) or the left border sequence in FIG. 6 (SEQ ID NO: 9) comprising genomic sugar beet DNA proximate the breakpoint of the insertion of the DNA into the genome and inserted plasmid DNA.

This invention encompasses a transgenic glyphosate resistant sugar beet wherein at least a 10–20 base pair fragment of said sequence of DNA is capable of detection by a pair of flanking primers wherein one of said primers is developed from the genomic sugar beet DNA proximate the breakpoint of the insertion of the DNA into the genome and one of said primers being developed from the inserted DNA plasmid.

#### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the primers used in the flanking PCR test.

FIG. 2 shows the diagrammatic interpretation of the Southern blot results.

FIG. 3 shows the plasmid sequence at the right border identified as SEQ ID NOS: 1, 2 and 3.

FIG. 4 shows the sequence of the right border breakpoint shown in SEQ ID NO: 4.

FIG. 5 shows the alignment of the right breakpoint region in the transformed sugarbeet T227-1 shown in SEQ ID NO: 6 with the untransformed plant allelic sequence shown in SEQ ID NO: 5.

FIG. 6 shows the plasmid sequence at the left border identified as SEQ ID NOS: 7, 8 and 9.

FIG. 7 shows the sequence of the left border breakpoint between the sugar beet genomic material and the inserted plasmid material shown in SEQ ID NO: 10.

FIG. 8 shows the alignment of the left breakpoint region shown in SEQ ID NO: 12 and the untransformed plant allelic sequence shown in SEQ ID NO: 11.

FIG. 9 shows the plasmid pMON17227.

FIG. 10a shows the sequence of the right border fragment obtained with the 98K89–RB12 primer set identified as SEQ ID NO: 13.

FIG. 10b shows the sequence of the right border fragment obtained with the 98K89–98I50 primer set identified as SEQ ID NO: 14.

FIG. 10c shows the sequence of the left border fragment obtained with the 98G94–98K86 primer set identified as SEQ ID NO: 15.

#### DETAILED DESCRIPTION OF THE INVENTION

The selection of a specific event in sugar beets (*Beta vulgaris*) requires the production of numerous transformants. These transformants must be screened in most instances to eliminate those transformants that do not conform fairly closely with the genotype of the original germplasm employed. These transformants must be tested for the efficiency of the inserted event. On a molecular basis these transformants must be tested for copy number to mostly to select for low copy number which may avoid gene silencing. Additionally, the transformants are tested and developed and screened based on the positional effect the location in the genome the inserted event has on the gene activity. Different insertion sites in the genome results in different overall results. For example, if the insertion is located in an area with high transcription there may be many copies of the protein produced; if the insertion is located in an area with low transcription, the protein may not be produced at sufficient levels for the desired gene effect. Thus, the selection of an insertion in recalcitrant plant such as sugar beet

involves numerous levels of investigative work and research. This type of research and development differs from plant to plant, from gene to gene, and according to the desired results.

The present invention broadly encompasses a sugar beet which is resistant to glyphosate at levels that would normally result in the death of the sugar beet plant. The resistance has been introduced by the *Agrobacterium* mediated transformation of the sugar beet with the pMON17227 plasmid. This plasmid is shown in FIG. 9 and it contains the FMV 35S promoter. The 35 S promoter is from a modified figwort mosaic virus. It also contains the chloroplast transit peptide from the EPSPS gene of *Arabidopsis*. And the CP4 synthetic gene optimized from the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene from *Agrobacterium* sp. Strain CP4. This plasmid also contains the terminator from pea. The terminator is from the pea gene RBCSE9. The E9 3' is the pea rbcS (small subunit ribulose biphosphate carboxylase oxygenase) E9 gene. The plasmid also includes the left and right T-DNA border sequences from *Agrobacterium*.

Numerous transformants were produced by the *Agrobacterium* mediated transformation of the sugar beet. The plantlets that survived the initial transformation stages were then screened in a green house for herbicide resistance. Levels of differing doses of herbicide were applied to the plant to determine which transformants were sensitive and which were resistant. The sensitive transformants were destroyed by the herbicide application and the resistant plants were subjected to further analysis based on comparison to the genome of the original germplasm employed in the transformation process. The transformants that were not eliminated in this screening were further selected from in further green house experiments. The resulting selected transgenic plants were analysed on a different criteria including on the molecular level.

This analysis led to the selection of a rare event insertion designated T227-1. The transformation method used to generate T227-1 integrated the vector into the genome by breakage of the circular vector at a position that was relative to the left and right border sequences. In the *Agrobacterium* system, the left and right border sequences are specifically recognised by vir genes (virD1 and virD2). These proteins produce nicks in the border sequences and the fragment is transferred from the bacterial cell into the plant cell as a single stranded T-DNA molecule, not as a circular vector.

To characterise T227-1 fully, as well as providing information that could serve as a basis for event-specific PCR tests, the nucleotide sequence of the breakpoints between inserted vector and plant genomic DNA was determined.

#### Flanking Sequences—T227-1

Genomic sequences flanking the T227-1 insert were isolated by linker PCR. Genomic DNA was digested with restriction endonucleases and linkers attached to the ends of the fragments obtained. The fragments containing the T227-1 sequences were preferentially amplified by PCR using the linker sequence and pMON17227-specific sequences as primers. The PCR products were sequenced directly, and the sequences used to design further primers, which were used to amplify plant genomic DNA from T227-1 and from untransformed plants.

Genomic plant DNA fragments were isolated for both left and right borders. These linker PCR products were sequenced and these sequences were aligned with (a) pMON17227 and (b) the fragments generated by the plant

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genomic primers using plant DNAs (containing the transformed and untransformed “alleles”) as templates.

Although the sequence contains some ambiguities, these are essentially consistent with the sequences being identical.

## Right Breakpoint

The present invention has its sugar beet genetic makeup at the insert sites in the right and left border regions identified and defined. In FIG. 3 the plasmid sequence at the right border is shown (SEQ ID NO: 2). The inserted DNA sequence was from pMON17227. The top portion of the drawing shows the sequence of the right border of the insert, the sugar beet genome and the beginning of the inserted foreign DNA (SEQ ID NO: 1). The Sub 105, the RB7 and the RB11 are small primer regions (nucleotides 5–22, 103–119 and 156–180, respectively, of SEQ ID NO: 2). The various combinations of primer regions can be employed singly or in pairs. The break at 7965 base pairs (indicated by the arrow) shows where the insertion has occurred. The DNA before this break is genomic sugar beet and after the break is the sequence from the plasmid.

FIG. 4 shows the sequence of the right border breakpoint and where the primers lie on this sequence (SEQ ID NO: 4). In the fourth row of sequence data the bolded sequence indicates the DNA sequence of the 98K89 primer (nucleotides 155–180 of SEQ ID NO: 4). The bolded sequence in the sixth line of sequence indicates the restriction site of BglIII (nucleotides 285–290 of SEQ ID NO: 4). The sequence that is in the greyed in area is the beginning of the integrated plasmid material from pMON17227 (nucleotides 364–400 of SEQ ID NO: 4). Within the greyed are two separate bolded portions of sequence. The first bolded sequence portion is the HindIII restriction site (nucleotides 370–375 of SEQ ID NO: 4). The second bolded sequence portion is the RB12 primer sequence (nucleotides 383–400 of SEQ ID NO: 4). The pair of primers, when employed, allow the PCR fragment of DNA that is also unique to this event to be generated.

FIG. 5 shows the alignment of the right breakpoint region in the transformed sugar beet T227-1 (SEQ ID NO: 6) with the untransformed plant allelic sequence (SEQ ID NO: 5). The sequence matches up so that it shows that the gene in the untransformed plant and the T227-1 event match. Certain base pairs could not be identified as ACTG and the following classification was used as listed in Table 1.

TABLE 1

S	= G or C
R	= A or G
W	= A or T
K	= G or T
M	= A or C
Y	= C or T
N	= any base
B	= C, G or T

There is a 17 base pair fragment (nucleotides 198–214 of SEQ ID NO: 6) in the last line of the sequence before the start of the plasmid sequence that indicates that there are 17 base pairs in the T227-1 transformed sugar beet that differs from the untransformed. After this 17 base pair fragment, which is in italics, the plasmid sequence data is found (nucleotides 215–240 of SEQ ID NO: 6).

The alignments of sequence data at the right side of the integration site are shown in FIG. 5. The nucleotides com-

## 6

mon to both the transformant (T227-1) and the plasmid (pMON17227) are indicated by an asterisk (\*) below the aligned sequence.

Between the vector breakpoint and the beginning of homology with beet genomic sequences, a 17 bp fragment is found which is not present in the untransformed allelic sequences studied in this germplasm. (nucleotides 198–214 of SEQ ID NO: 6). No perfect homology could be found between this short unknown DNA sequence and the plasmid sequence.

These results are entirely consistent with the Southern and PCR analyses of T227-1.

## Left Breakpoint

FIG. 6 shows the plasmid sequence at the left border (SEQ ID NO: 9). The inserted DNA sequence was from pMON17227. The top portion of the drawing shows the sequence of the left border of the insert and the associated sugar beet genome (SEQ ID NO: 7). The base pair 2336 is a portion of the left border of the inserted DNA material (SEQ ID NO: 8). The Sub 103 (nucleotides 13–30 of SEQ ID NO: 8), Sub 104 (nucleotides 134–150 of SEQ ID NO: 9), LB8 (nucleotides 21–36 of SEQ ID NO: 9) and the LB9 (nucleotides 81–98 of SEQ ID NO: 9) are small primer regions. The break at 2686 base pairs (indicated by the arrow) shows where the insert border has occurred. The DNA before this break is the sequence from the plasmid and after the break is genomic sugar beet.

FIG. 7 shows the sequence of the left border breakpoint between the sugar beet genomic material and the inserted plasmid material (SEQ ID NO: 10). The greyed in region is the integrated plasmid sequence (nucleotides 1–143 of SEQ ID NO: 10). At the end of this sequence is the bolded primer LB8 (nucleotides 128–143 of SEQ ID NO: 10). In the next line is the bolded sequence that indicates the restriction site for BglIII (nucleotides 181–186 of SEQ ID NO: 10). The primer that is located in the sugar beet genomic material is identified in bold in the figure at the bottom (nucleotides 730–753 of SEQ ID NO: 10).

FIG. 8 shows the alignment of the left breakpoint region (SEQ ID NO: 12) and the untransformed plant allelic sequence (SEQ ID NO: 11). The T227-1 transformed event starts with the plasmid sequence. Then there is a region of sparse matching between the untransformed and the T227-1, a fragment of 49 base pairs (nucleotides 10–58 of SEQ ID NO: 12). Thereafter there is a close correlation between the untransformed sugar beet and the transformed sugar beet called event T227-1.

The alignments of sequences from the left side of the integration site and its known component elements are shown in FIG. 8.

At the 3' end of the sequence there is clear homology to the pMON17227 vector though there is a 49 base pair fragment (nucleotides 10–58 of SEQ ID NO: 12) that sparsely aligns with the untransformed beet “allele”, or to pMON17227.

On the basis of the sequence shared by the transformant and the plasmid it is clear that a 4 base pair section of the left T-DNA border was incorporated into the Event T227-1 during or following the transformation event. No right T-DNA border and very little of the left T-DNA border portions, were integrated into the transgenic sugar beet event entitled T227-1.

Beyond this 4 base pair section of the left T-DNA border region from *Agrobacterium* the plasmid sequence has been entirely integrated, excluding the right border section of the



*Agrobacterium* region, after which point recombination has taken place to insert the construct into the beet genome.

These results are fully consistent with the Southern hybridisation results.

#### Use of Flanking Primers to Analyse T227-1 and Untransformed Sugar Beets

Specific primers were developed from the left and right flanking sequences, and used in PCR reactions with each other or individually with primers from within the insert. Primer locations in the inserted DNA and in the sequence flanking the insert are shown in FIGS. 1, 3–7 and Table 1.

PCR reactions between a flanking primer and an insert primer always gave the size expected from the sequence information, and have never given any product in untransformed beet or transformants other than T227-1. Thus these sequences are used as an event-specific test whereby the T227-1 event is identified. This identification process of the insertion of event T227-1 which is one copy and has little extraneous DNA and is highly efficacious at resisting the effects of glyphosate on the plant, is possible in the original germplasm and in progeny developed by any manner of breeding or selfing. The material from the progeny including pollen and seeds and the like can be tested for the presence of this insert in the DNA.

#### Genomic Flanking Sequences

The plasmid shown in FIG. 9 contained the LB (left border) RB (the right border), the FMV 35S promoter (from a modified figwort mosaic virus), and the main gene of interest to give glyphosate resistance the CP4 syn: the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene from *Agrobacterium* sp. Strain CP4. The plasmid also contains a chloroplast transit peptide and a terminator from pea. The circular vector pMON17227 opens at both left and right borders when inserted into the plant genome to form the transformed Event. The selected transformation event was the T227-1 Event. This transformed beet T227-1 and its progeny show excellent levels of glyphosate tolerance.

To characterize T227-1 fully, providing information that serves as a basis for event-specific PCR tests, the nucleotide sequences of the breakpoints between inserted vector and plant genomic DNA were determined.

Genomic sequences flanking the T227-1 insert were isolated by linker PCR. Genomic DNA was digested with restriction endonucleases and linkers attached to the ends of the fragments obtained. The fragments containing the T227-1 sequences were preferentially amplified by PCR using the linker sequence and pMON17227-specific sequences as primers. The PCR products were sequenced directly, and the sequences used to design further primers; these were then used to amplify plant genomic DNA from the T227-1 transformant for both left and right borders (flanking sequences PCR) and from an untransformed plant.

FIG. 1 shows the primers used in the flanking PCR test. The first pair of primers is in the right border region of the inserted material. 98k89 (nucleotides 155–180 of SEQ ID NO: 4) is a primer that is located within the sugar beet plant genome. 98I50 (nucleotides 455–476 of SEQ ID NO: 14) is a primer that is located in the inserted plasmid DNA sequence. In combination these primers can be employed to produce a unique piece of DNA through a PCR test. This unique DNA specifically identifies this T227-1 transforma-

tion event. The sugar beet identified by this PCR flanking sequence is only the T227-1 event or its progeny.

The second pair of primers is in the left border region of the inserted material. 98K86 (nucleotides 730–753 of SEQ ID NO: 10) is a primer that is located within the sugar beet plant genome. 98G94 (nucleotides 1–20 of SEQ ID NO: 15) is a primer that is located in the inserted plasmid DNA sequence. In combination these primers can be employed to produce a unique piece of DNA through a PCR test. These event-specific tests using the primers include a method that employs electrophoresis through gels as the standard method used to separate, identify, and purify DNA fragments. The location of the DNA that is separated by size within the gels can be determined by using a dye. This dye permits DNA bands with 1–10 ng of DNA to be detected under ultraviolet light. This unique DNA specifically identifies the T277-1 transformation event. The sugar beet identified by this PCR flanking sequence is only the T277-1 event or its progeny. Test kits comprising at least one set of primers wherein one such primer is located within the sugar beet plant genome proximate the insert and the other such primer is located within the inserted plasmid DNA sequence of T227-1, wherein a unique piece of DNA is capable of being identified wherein the material is identified as being GMO positive or GMO negative. Additionally, the material can be more specifically identified as being T277-1 or its progeny.

Primers 98K89 (nucleotides 155–180 of SEQ ID NO: 4) and 98K86 (nucleotides 730–753 of SEQ ID NO: 10) for the T277-1 event or its progeny are situated in the regions flanking the inserted sequences, while 98I50 (nucleotides 455–476 of SEQ ID NO: 14) and 98G94 (nucleotides 1–20 of SEQ ID NO: 15) lie inside the DNA from the vector. The resulting PCR products are described in Table 2.

TABLE 2

Specific primers inside and outside the integrated vector sequence.

	Vector specific primer (position)	Flanking DNA primer	PCR product
Left border	98G94 (2503 bp) (nucleotides 1–20 of SEQ ID NO: 15)	98K86 (nucleotides 730–753 of SEQ ID NO: 10)	792 bp (SEQ ID NO: 15)
Right border	98I50 (8232 bp) (nucleotides 455–476 of SEQ ID NO: 14)	98K89 (nucleotides 155–180 of SEQ ID NO: 4)	476 bp (SEQ ID NO: 14)

PCR with these primers was performed on a 9600 thermocycler from PE-Biosystems. In each 50 µl reaction, 50 ng of plant DNA was incubated with 120 ng of each primer and 1U of Amplitaq DNA Polymerase in GeneAmp PCR buffer II containing 10 mM Tris-HCl pH8.3 and 50 mM KCl complemented with 1.5 mM MgCl<sub>2</sub> and 0.2 mM dNTP. One PCR reaction consisted of a hot start of 3 min. at 94° C. followed by 35 cycles (one cycle: 30 sec. at 94° C., 1 min. at 57° C. and 1 min. at 72° C.). The PCR products were separated on a 1.5% agarose gel.

This amplification clearly demonstrates that the left and right flanking sequences are normally contiguous in sugar beet, and have not suffered major reorganisation as a result of the transformation.

#### Flanking Sequences—Progeny of T227-1

By sequencing the flanking regions we developed specific primers from the left and right flanking sequences, and used

these in PCR reactions with each other or individually with primers from within the insert. Such a PCR assay can be used to identify the T227-1 transformation event and its progeny uniquely.

Primer pairs (plant genomic DNA/plasmid DNA) for both the left and the right region were tested on T227-1, its progeny and the non-transformed lines. Both primer combinations gave positive results for all the glyphosate resistant plants (all generations). All these PCR products were identical to those obtained with transformation event T227-1. The fragment lengths of a few of the combinations are listed in FIGS. 10a–10c (SEQ ID NOS: 13–15). Other primer pairs can give different fragments. The glyphosate sensitive plants and the non-transformed line, however, showed no amplification product at all.

The T227-1 event and its progeny were tested for this unique DNA by using Southern blots. FIG. 2 shows the diagrammatic interpretation of the Southern blot results. The four boxes on top of the figure depict the plasmid sequence DNA that was incorporated into the transformed sugar beet T227-1. The components of the plasmid differ somewhat from the inserted material. The sugar beet was transformed through the use of *Agrobacterium*. Therefore, the plasmid contained both the right and left T-DNA border regions. The right T-DNA border sequence was not transformed into T227-1. The left T-DNA border sequence was not transformed except for a small 4 base pair section that is integrated between the sugar beet plant genome and the E9 3' terminator sequence.

The inserted material is identified as FMV, CTP 2, CP4 syn, E9 3', LB. FVM is a 35S promoter from a modified figwort mosaic virus. CP4 syn is the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene from *Agrobacterium* sp. Strain CP4. CTP2 is the chloroplast transit peptide from *Arabidopsis*. E9 3' is the terminator. LB is the small portion of the left border sequence from *Agrobacterium*.

The vertical arrows depict the restriction sites located in the genome and the inserted material. The horizontal lines below the top two boxes depict the approximate number of base pairs that are in a fragment that is produced by employing the restriction enzyme indicated on the top box. The second box depicts the transformed sugar beets genetic makeup at the insert site.

The beet genomic sequences flanking this insert have been sequenced, and employed to design specific PCR tests for the T227-1 insertion. Flanking primers may be used for amplification of untransformed sugar beet DNA, indicating that the transformation did not induce any major rearrangements in the beet genome. This is confirmed by sequence analysis, which shows that the sequences on either side of the insert are essentially collinear with those of untransformed "alleles" from the same locus. Thus the present invention includes a method of using primers that identify T227-1 in a PCR method of detecting DNA. The invention also covers each of the two pair of primers that flank the right and left borders. The invention further covers the sugar beet having the DNA that is detectable by the primers.

## SEQUENCE LISTING

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<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Plasmid sequence at the right border

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ctcaggattt agcagcattc 200

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<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

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<220> FEATURE:  
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 tgtcatgatt caacaccaa tgacacttaa attaagaacc cacctcatct taaaccaaac 180  
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 tttatTTTTc aatcaagatc tatacaacta ataactgaag taggagaaga tatgggattg 180  
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 <223> OTHER INFORMATION: Description of Artificial Sequence: Plasmid  
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&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Plasmid  
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&lt;211&gt; LENGTH: 160

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Plasmid  
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&lt;211&gt; LENGTH: 797

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Sequence of  
the left border breakpoint

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&lt;221&gt; NAME/KEY: misc\_feature

&lt;222&gt; LOCATION: 6, 21, 53, 90, 108, 136, 164, 169, 318, 778..780

&lt;223&gt; OTHER INFORMATION: n = any base

&lt;400&gt; SEQUENCE: 10

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atttcccga catgangcca ttcccatat ctctcctac ttcnagtcna ttagttgtat 180

agatcttgat tgaaaaataa atatttgctc caactctctt ttattccctg tgtccatgctc 240

tgaacaactt tcgaattttc ttcctaataa tctcgcgata acttgcagtg tttggaacat 300

gcaatgagcg agaaatanaa attttatttc tgctttgaaa gcaattgta gaatgcatcg 360

tcctactgtg attgcatgag tggaaacaca tatgggagga aatcaagcta tgtctattgc 420

atctgctctg gggactctg gtcatactcg tgcgatgcc atgggttttt tagggggaat 480

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ggagatggct tcatgtccat gtgtttattc ccatcaagct tgagctcagg atttagcagc	240
attccagatt gggttcaatc aacaaggtac gagccatc actttattca aattggtatc	300
gccaaaacca agaaggaact cccatcctca aaggtttga aggaagaatt ctcagtccaa	360
agcctcaaca aggtcagggt acagagtctc caaaccatta gccaaaagct acaggagatc	420
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<220> FEATURE:  
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 <223> OTHER INFORMATION: n = any base

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ttctccatat tgaccatcat actcattgct gatccatgta gatttcccgg acatgaagcc	180
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aatatttgtc ccaactctct tttattccct gtgtccatgt ctgaacaact ttcgaatttt	300
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aattttatatt ctgctttgaa agcaattggt agaatgcatc gtcctacgtg attgcatgag	420
tggaacaca tatgggagga aatcaagcta tgtctattgc atctgctctg ggtactctg	480
gtcactactcg tgtcagatgcc atgggttttt tagggggaat tttgatttat tggaacaga	540
attggttacc atagaacctc tcattagaca tgcatgatca acatataacc atggaaataa	600
aaagggtagg ggctattctt tggattttct cagcggttta tgcgagtccc gaccctaaa	660
aacgccaaagt tctttggcaa gaattaagaa atttcgctcg aactcataat caagcttggc	720
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aaactcaacg tt	792

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We claim:

1. A pair of primers selected from the group consisting of: the pair of primers identified as 98G94, which consists of nucleotides 1–20 of SEQ ID NO: 15 and 98K86, which consists of nucleotides 730–753 of SEQ ID NO: 10 and;  
the pair of primers identified as 98I50, which consists of nucleotides 455–476 of SEQ ID NO: 14 and 98K89, which consists of nucleotides 155–180 of SEQ ID NO: 4.
2. A pair of primers of claim 1 that hybridizes to a transgenic insert of DNA of a transgenic glyphosate-resistant sugar beet, wherein the transgenic insert of DNA comprises at least one right border region, and at least one left border region, and comprises a figwort mosaic virus 35S promoter, DNA encoding a chloroplast transit peptide from the EPSPS gene of *Arabidopsis*, and an optimized CP4 EPSPS gene from *Agrobacterium* sp. CP4, and a terminator.
3. A pair of primers according to claim 2 comprising DNA which hybridizes to DNA that lies in the right border region of the insert DNA.
4. A pair of primers according to claim 2 comprising DNA which hybridizes to DNA that lies in the left border region of the insert DNA.
5. A pair of primers according to claim 3 or claim 4, wherein one primer of the pair comprises DNA which

hybridizes to genomic DNA of the transgenic beet and the other primer hybridizes to the transgenic insert.

6. A method of detecting a transgenic sugar beet having an insert DNA comprising a figwort mosaic virus 35s promoter, a chloroplast transit peptide from the EPSPS gene of *Arabidopsis*, an optimized CP4 EPSPS gene from *Agrobacterium* sp. strain CP4 and a terminator; the method comprising the steps of:

- 1) amplifying DNA from the transgenic beet in the presence of a pair of primers selected from the group consisting of:

the pair of primers identified as 98G94, which consists of nucleotides 1–20 of SEQ ID NO: 15 and 98K86, which consists of nucleotides 730–753 of SEQ ID NO: 10 and;

the pair of primers identified as 98I50, which consists of nucleotides 455–476 of SEQ ID NO: 14 and 98K89, which consists of nucleotides 155–180 of SEQ ID NO: 4

using the polymerase chain reaction and

- 2) detecting the presence or the absence of the DNA fragment.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 7,241,567 B2  
APPLICATION NO. : 10/415602  
DATED : July 10, 2007  
INVENTOR(S) : G. Weyens et al.

Page 1 of 2

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

<u>COLUMN</u>	<u>LINE</u>	<u>ERROR</u>
19 (Claim 1, line 3)	4	“15” should read --15,--
19 (Claim 1, line 4)	5	“NO: 10” should read --NO: 10;--
19 (Claim 1, line 5)	6	“and;” should read --and--
19 (Claim 1, line 7)	8	“14” should read --14,--
20 (Claim 6, line 3)	5	“chioroplast” should read --chloroplast--
20 (Claim 6, line 11)	14	“15” should read --15,--
20 (Claim 6, line 12)	15	“NO: 10” should read --NO: 10;--
20 (Claim 6, line 13)	16	“and;” should read --and--
20 (Claim 6, line 15)	18	“14” should read --14,--
20 (Claim 6, line 17)	20	“4” should read --4;--
20 (Claim 6, line 18)	21	“polyrnerase” should read --polymerase--



UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 7,241,567 B2  
APPLICATION NO. : 10/415602  
DATED : July 10, 2007  
INVENTOR(S) : G. Weyens et al.

Page 2 of 2

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

<u>COLUMN</u>	<u>LINE</u>	<u>ERROR</u>
20 (Claim 6,	21 line 18)	“reaction and” should read --reaction; and--

Signed and Sealed this

Eighteenth Day of December, 2007

A handwritten signature in black ink on a dotted background. The signature reads "Jon W. Dudas" in a cursive style.

JON W. DUDAS

*Director of the United States Patent and Trademark Office*